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Rapid and efficient stem segment regeneration propagation system for *Euonymus bungeanus*

Kai-Ge Cui
Agricultural College of Yanbian University
Hao-Lin Wang
Agricultural College of Yanbian University
Wen-Yue Quan
Agricultural College of Yanbian University
Zhuo-Zhuo Ye
Agricultural College of Yanbian University
Cheng-Ri Zhao
zhaochengri@ybu.edu.cn

Agricultural college of Yanbian University https://orcid.org/0000-0001-8362-1125

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Abstract

Euonymus bungeanus Maxim. is a tree species with high ornamental, industrial, and medicinal value. Establishing a method for rapid and efficient regeneration of *E. bungeanus* is essential to achieve industrial-scale production. The aim of this study was to establish a rapid tissue propagation technique for *E. bungeanus* and provide a foundation for the industrial production of tissue-cultured seedlings. Using stem segments of *E. bungeanus* as explants, we investigated effects of explant collection time, sterilization method, various culture media, and ratios of plant growth regulators on the initiation, subculture, and rooting stages of the tissue culture process for *E. bungeanus*. The optimal explant collection time was mid-April; a combination of 75% ethanol for 20 s, followed by 0.1% $HgCl_2$ for 7 min was suitable for disinfection, yielding a survival rate of 55.00% for the explants. Initiation culture using the woody plant medium (WPM) supplemented with 1.0 mg L^{-1} of 6-benzylaminopurine (6-BA) and 0.2 mg L^{-1} of α-naphthalene acetic acid (NAA) achieved an induction rate of 87.22% for explants. Proliferation culture on ¼ WPM medium containing 2.0 mg L^{-1} of 6-BA and 0.1 mg L^{-1} of NAA resulted in a propagation coefficient of 4.98. Rooting culture on ½ Murashige and Skoog (MS) medium supplemented with 0.2 mg L^{-1} of indole-3-butyric acid achieved a rooting rate of 78.33%. However, the rooting rate was low, indicating the need for further optimization of rooting and acclimatization. This study is the first to develop a rapid and efficient regeneration system for *E. bungeanus* using stem segments.

Introduction

Euonymus bungeanus Maxim is a deciduous small tree belonging to the *Euonymus* L. genus in the Celastraceae family. It can grow to 8 m in height and is widely distributed throughout China (Du et al. 2016; Thomas et al. 2011). Its distribution spans northern regions, including Heilongjiang, Jilin, Liaoning, North China, and Inner Mongolia, to southern regions along both sides of the Yangtze River but not in Guangdong or Guangxi. It is also found in the Ussuri region, southern Siberia, and the Korean Peninsula (Thomas et al. 2011; Zhu et al. 2012).

Euonymus bungeanus has high ornamental value and resistance (Li et al. 2022). For example, its leaves turn red in autumn, and its branches are adorned with abundant autumn fruits that split open to reveal orange-red arils. It exhibits drought resistance, cold tolerance, waterlogging endurance, pruning tolerance, and resistance to pests and diseases. Furthermore, it thrives in neutral, acidic, and alkaline soils and can grow in mildly saline-alkaline soils. It has a strong dust-retaining capability and exhibits robust resistance to harmful gases, such as sulfur dioxide, improving air quality (Wang et al. 2022; Durrett et al. 2010). Therefore, gardening and landscaping enthusiasm has gained increasing attention as highly resilient ornamental tree species with colorful leaves. In contrast, *E. bungeanus* has industrial and medicinal value. Its wood is tough, fine-textured, clean white, and suitable for carving and crafting various items, such as sculptures, booms, and tackles (Zhu et al. 2012; Wu et al. 2021). The root and tree bark contains *Eucommia ulmoides* gum, which can be used as an industrial raw material (Li et al. 2020; Durrett et al.

2010). The seeds have a high oil content, exceeding 40% (Liu et al. 2019; Mihálik et al. 2020). Its seeds, roots, leaves, and fruits can be used to treat conditions like knee joint pain and skin ulcers (Fu et al. 2019; Liu et al. 2019). The seeds are also rich in fatty substances (Liu et al. 2019).

Seed propagation is a commonly used sexual reproductive method in *E. bungeanus*. Because of the phenomenon of trait segregation in the progeny resulting from sexual reproduction, ensuring the consistency and stability of traits becomes challenging. Therefore, an extended purification process is necessary before achieving stability, and the stabilized offspring can be used for production purposes. However, asexual reproduction involves regenerating a complete plant from somatic cells or portions of plant tissue. Genetic information is transmitted by replicating genetic material in meristematic tissue cells, resulting in individuals identical to the parent plant. Among the methods of asexual reproduction, plant tissue culture stands out owing to its rapid propagation, scalability, cost-effectiveness, spatial efficiency, and seasonal influence. These characteristics make it highly suitable for the large-scale production of ornamental plants in landscaping (Smith and Jernstedt 1989; Bhojwani and Dantu 2013).

Establishing a plant tissue culture system encompasses selection of explants, choice of differentiation pathways, selection of culture media, and determination of the types and concentrations of plant growth regulators. Research indicates that the selection of explants is a critical factor in determining the success of plant tissue culture because of variations in the genotype, types, concentrations of plant growth regulators, and other factors among different plant sources (Dobránszki and Da Silva 2010). The type of explant, seedling age, sampling site, sampling season, and growth environment influence the establishment of a plant tissue culture system. Typical woody plant explants include the stem segments, gametophytes, embryos, and leaves. For instance, *Malus* commonly utilizes stem segments, leaves, and seeds (Alayón-Luaces et al. 2008); *Vaccinium* spp. often employs leaves, nodal stem segments, and axillary buds (Paprštein and Sedlák 2015); *Dalbergia congestiflora* Pittier frequently utilizes stem segments (Ali et al. 2012).

This study utilized nodal stem segments from cold-resistant wild *E. bungeanus* from the Changbai Mountains region of Jilin Province, China, as explants to establish a rapid tissue propagation technique for *E. bungeanus*. This study aimed to provide a reference for improving the commercial production of *E. bungeanus*. This research fills a gap in knowledge using *E. bungeanus* stem segments as explants to generate a tissue culture system.

Materials and methods

Plant materials

Plant materials were obtained from 2–3-year-old *E. bungeanus* plants at Longjing City Forestry Farm (129.419722°E, 42.785278°N) in Jilin Province, China. Well-developed branches from the current year were selected from healthy parent trees for use as experimental materials.

Experimental methods

Initiation culture of stem segments

Explant sterilization

Euonymus bungeanus branches collected during different months (mid-March to mid-September) were selected for further study. After removing the leaves and leaf stalks, the branches were washed with diluted washing liquid using a brush to clean the surface. The branches were cut into 3–4 cm long segments with axillary buds and rinsed under running water for 1–2 h. The segments were then transferred to a clean bench for sterilization. First, they were immersed in 75% ethanol (Lilkang Med-Tech Co., Ltd., Shandong, China) for 30 s for surface disinfection. Then, they were treated with 0.1% $HgCl_2$ (Shanghai Macklin Biochemical Co., Ltd., Shanghai, China) for 7 min. Finally, the segments were rinsed 5 times with sterile water. After disinfection, stem segments were transferred onto sterile filter paper to remove excess surface moisture. Then, approximately 0.5 cm from both the upper and lower parts of the segments (where the segments had direct contact with the disinfectants) were removed.

The stem segments were then inoculated onto MS (Murashige and Skoog 1962) culture medium, with 20 bottles per treatment and three replicates, each bottle containing two explants. After 15 d of cultivation, contamination, survival, and mortality rates were recorded. The cultivation conditions were maintained at 25 ± 2°C, with a humidity of 50–60%, a photoperiod of 12 h per day, and a light intensity of 2000–2500 lx (the same below).

Contamination rate (%) = (Number of explants contaminated/Total number of explants inoculated) × 100% (Wu et al. 2023; Gu et al. 2022; Suraya et al. 2021; Cuba-Díaz et al. 2020). (1)

Survival rate (%) = (Number of explants that survived/Total number of explants inoculated) × 100% (Han et al. 2023; Wu et al. 2023; Zhang et al. 2022). (2)

Mortality rate (%) = (Number of explant mortality/Total number of explants inoculated) × 100% (Wang et al. 2018). (3)

Effect of different disinfection methods on explant sterilization efficacy

The branches of *E. bungeanus* collected in mid-June were stripped from the leaves and stalks. They were then cleaned using a brush dipped in a diluted washing liquid solution to remove surface contaminants. Subsequently, the branches were cut into 3–4 cm long segments with axillary buds and rinsed under running water for 1–2 h. The segments were then transferred to a clean bench for sterilization. In this study, a combination of alcohol disinfection followed by either NaClO (Shanghai Macklin Biochemical Co., Ltd., Shanghai, China) or $HgCl_2$ disinfection was used as an explant sterilization method.

To determine the optimal duration of alcohol disinfection, the following three disinfection combinations were used:

(1) immersed in 75% ethanol for 10 s, followed by immersion in 0.1% $HgCl_2$ for 5 min.

(2) immersed in 75% ethanol for 20 s, followed by immersion in 0.1% $HgCl_2$ for 5 min.

(3) immersed in 75% ethanol for 30 s, followed by immersion in 0.1% $HgCl_2$ for 5 min.

To determine the optimal effect of NaClO or $HgCl_2$ disinfection after alcohol disinfection, the following eight disinfection treatment combinations were used:

(1) immersed in 75% ethanol for 20 s, followed by immersion in 1% NaClO for 10 min.

(2) immersed in 75% ethanol for 20 s, followed by immersion in 1% NaClO for 20 min.

(3) immersed in 75% ethanol for 20 s, followed by immersion in 2% NaClO for 10 min.

(4) immersed in 75% ethanol for 20 s, followed by immersion in 2% NaClO for 20 min.

(5) immersed in 75% ethanol for 20 s, followed by immersion in 0.1% $HgCl_2$ for 5 min.

(6) immersed in 75% ethanol for 20 s, followed by immersion in 0.1% $HgCl_2$ for 6 min.

(7) immersed in 75% ethanol for 20 s, followed by immersion in 0.1% $HgCl_2$ for 7 min

(8) immersed in 75% ethanol for 20 s, followed by immersion in 0.1% $HgCl_2$ for 8 min

After each treatment, the segments were rinsed five times with sterile water. The disinfected stem segments were air dried by blotting with a sterile filter paper to remove surface moisture, and approximately 0.5 cm of the segment’s upper and lower portions were excised. They were then inoculated onto a hormone-free MS culture medium. Each treatment included 20 bottles, with two explants per bottle and three replicates. After 20 d of cultivation, contamination, survival, and mortality rates were recorded.

Effect of different culture media on the induction of stem segments

After being soaked in 75% ethanol for 20 s and immersed in 0.1% $HgCl_2$ for 7 min, aseptic stem segments were obtained and inoculated into Gamborg's B5 (B5) (Gamborg et al. 1968), MS, and woody plant medium (WPM) (McCown 1981) culture media. The B5, MS, and WPM culture media (were as below):80 mL per bottle, pH 5.8, and autoclaved at 121 °C under high-pressure steam for 20 min. Each culture medium was supplemented with 6-BA (Shanghai Yuanye Bio-Tech Co., Ltd., Shanghai, China) 1.0 mg L^{-1} + NAA (Yuanye Bio-Tech Co., Ltd, Shanghai, China) 0.1 mg L^{-1} + agar (Beijing Aoboxing Bio-Tech, Beijing, China) 7 g L^{-1} + sucrose (Tianjin Kermel Chemical Reagent Co., Ltd., Tianjin, China) 30 g L^{-1}. Twenty bottles were used for each culture medium, with three replicates, and each bottle contained two explants. After 30 d of cultivation, the rate of axillary bud induction was recorded.

Induction rate (%) = (No. of explants that induced buds/No. of explants inoculated) × 100% (Amiri and Mohammadi 2021; Cui et al. 2019) (4)

Effect of different kinds, concentrations, and ratios of plant growth regulators on the induction of stem segments

The obtained sterile stem segments were inoculated using the abovementioned method onto a WPM culture medium containing different types and concentrations of plant growth regulators. Cytokinin 6-BA and auxin NAA were set at three levels each: 6-BA concentrations of 1.0 mg L^{-1}, 1.5 mg L^{-1}, and 2.0 mg L^{-1}; NAA concentrations of 0.1 mg L^{-1}, 0.2 mg L^{-1}, and 0.3 mg L^{-1}. Nine treatment combinations were created from the two factors with three levels each, and a control treatment without any plant growth regulators was included. Each treatment was inoculated into 20 bottles with three replicates and three explants per bottle. After 30 d of cultivation, the induction rate of axillary buds was recorded, and the growth of tissue-cultured seedlings was observed.

Subculturing of stem segments

Effect of different media concentrations on stem segment proliferation

After successful axillary bud induction, 2–3 cm shoots were transferred to different concentrations of WPM culture medium to observe their propagation. Three different concentrations of WPM were used: WPM, ½ WPM, and ¼ WPM. Each medium was supplemented with 6-BA 1.0 mg L^{-1} + NAA 0.1 mg L^{-1} + agar 7 g L^{-1} + sucrose 30 g L^{-1}. Each medium was inoculated into 20 bottles, with two explants per bottle, and the experiment was repeated three times. After 30 days of cultivation, the propagation status was assessed and recorded.

Proliferation coefficient = No. of final buds/No. of initial buds (Amiri and Mohammadi 2021; Cui et al. 2019) (5)

Effect of different kinds, concentrations, and ratios of plant growth regulators on the proliferation of stem segments

Well-developed and similarly sized axillary buds were chosen and transferred to culture media with different combinations and concentrations of plant growth regulators for subculture. Cytokinin 6-BA and auxin NAA were set at three levels each: 6-BA concentrations of 1.0 mg L^{-1}, 2.0 mg L^{-1}, and 3.0 mg L^{-1}; NAA concentrations of 0.1 mg L^{-1}, 0.2 mg L^{-1}, and 0.3 mg L^{-1}. This formed a 2-factor, 3-level design, resulting in nine treatment groups. Each treatment was inoculated into 20 bottles with three replicates and two explants per bottle. After 40 days of cultivation, the propagation status was assessed.

Rooting culture

Effect of different culture media on rooting

Vigorous plantlets were selected from aseptic plantlets obtained by subculturing the stem segments. Basal segments of non-rooted shoots measuring 1–2 cm and bearing 3–5 leaves were excised. These segments were then inoculated onto solid culture media containing indole-3-butyric acid (IBA) (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at a 0.1 mg L^{-1}. Four types of solid culture media were used: MS, WPM, ½ MS, and ½ WPM. Each type of culture medium was used to inoculate 20 bottles with three replicates per treatment and two explants per bottle. After 40 d of cultivation, the root formation of the explants was assessed, and the rooting rate was calculated.

Rooting rate (%) = No. of rooting plants/No. of inoculated plantlets × 100% (Amiri and Mohammadi 2021; Fernandes et al. 2020; Shaik et al. 2018) (6)

Effect of different rooting agents and concentrations on rooting

Vigorous plantlets were selected from aseptic plantlets obtained by subculturing the stem segments. Basal segments of non-rooted shoots measuring 1–2 cm and bearing 3–5 leaves were excised and inoculated on ½ MS culture medium containing different rooting agents at different concentrations. When using a single rooting agent, the concentrations of NAA were set at 0.2 mg L^{-1}, 0.4 mg L^{-1}, and 0.6 mg L^{-1}; the concentrations of IBA were set at 0.2 mg L^{-1}, 0.4 mg L^{-1}, and 0.6 mg L^{-1}. When using a combination of two rooting agents, the concentrations of NAA and IBA were set at 3 levels each (NAA: 0.05 mg L^{-1}, 0.1 mg L^{-1}, 0.2 mg L^{-1}; IBA: 0.2 mg·L^{-1}, 0.4 mg L^{-1}, and 0.6 mg L^{-1}). Fifteen treatments were developed by combining these factors. Each treatment was inoculated into 20 bottles, with three replicates per treatment and two explants per bottle. After 40 d of cultivation, the rooting conditions were assessed, and the rooting rate was calculated.

Statistical analysis

Microsoft Excel 2019 (Microsoft Corp., Redmond, WA, USA) and IBM SPSS Statistics 26 (IBM Corp., Armonk, NY, USA) were used for data processing. The means were compared using Duncan's multiple-range test at a 5% level.

Results

Stem initiation culture

Optimal sampling time selection

The contamination rate of explants collected in different mid-months showed an increasing trend with an increase in months. The contamination rate of explants from mid-September almost reached 100% (Table 1). This indicates that the longer the exposure time of the plant material under natural environmental conditions, the more severe the contamination of the explants. Analysis of the mortality rate revealed that the highest mortality rate was observed in explants collected in mid-March. This indicates that, although the contamination rate of the spring shoots in mid-March was very low, the tender nature of the explants and their weaker resistance to disinfectants resulted in the blackening and death of the inoculated explants. Analysis of the survival rate indicated that the highest survival rate was observed in explants collected in mid-April among the analyzed samples. Therefore, considering the contamination, mortality, and survival rates, the optimal collection time was determined to be mid-April. During this period, the contamination rate of the explants was at its lowest, the mortality rate was relatively low, and the survival rate was at its highest.

Screening for the best stem segment disinfection method

In this study, stem segment disinfection was carried out using a combined approach of first immersing the segments in 75% ethanol for disinfection, followed by subsequent immersion in $HgCl_2$ or NaClO solutions.

The experimental results indicated that the contamination rate decreased with increasing ethanol disinfection time. The mortality rate was relatively low for the 10 and 20-s treatments, reaching its highest value after 30 s. The survival rate was lower for both the 10 and 30-s treatments, with the highest value observed for the 20-s treatment (Table 2). This suggests that shorter ethanol disinfection times resulted in insufficient disinfection and higher contamination rates. However, excessively prolonged disinfection could damage explants or even lead to death. The optimal ethanol disinfection time was determined to be 20 s, considering the contamination, mortality, and survival rates. At this time, the contamination rate of the explants was relatively low, the mortality rate was minimal, and the survival rate was the highest.

Secondly, to determine the choice between $HgCl_2$ and NaClO as the subsequent disinfectant, stem segments first soaked in 75% ethanol for 20 s were subjected to eight treatments (Table 3). The results indicated that the disinfection effect of NaClO was not as effective as $HgCl_2$. Specifically, treatment with 1% NaClO for 10 or 20 min resulted in 100% contamination rates. The use of 2% NaClO treatment showed a decreasing trend in contamination rate, but even the lowest value reached 87.50%, with a survival rate of only 7.50%. In contrast, when using 0.1% $HgCl_2$ immersion disinfection, the contamination rate showed a noticeable decrease with extended disinfection time. The lowest contamination rate was achieved at the 8-min disinfection treatment, reaching 22.50%. The effect of $HgCl_2$ treatment between 5 and 7 min on the relatively minor mortality rate remained at a low level between 7.50–12.50%. However, with prolonged treatment time, the mortality rate trended upward (e.g., after the 8 min disinfection treatment, the mortality rate increased to 25.00%). Different treatment durations showed relatively high survival rates, ranging from 32.50 to 55.00%, with the highest value at the 7-min treatment.

Considering the contamination, mortality, and survival rates, the optimal method for stem segment sterilization was first soaking them in 75% ethanol for 20 s, followed by a 7-min exposure to 0.1% $HgCl_2$.

Selection of primary culture medium

To determine the optimal primary culture medium for *E. bungeanus*, sterile stem segments were inoculated onto the three aforementioned solid culture media, and the effects of different media on axillary bud induction were observed (Table 4, Fig. 1). The experimental results showed that all three culture media were capable of inducing axillary bud sprouting. However, the WPM culture medium exhibited a significantly higher axillary bud induction rate compared to the other two media, reaching up to 78.33%. In addition, plants grown on WPM displayed the best growth, with vibrant green leaves. Conversely, seedlings cultured on the MS medium exhibited weaker growth, with the appearance of callus tissue at the base and slow development. Seedlings grown on B5 medium displayed gradual yellowing and wilting of the leaves.

Therefore, considering the induction rate and growth performance, WPM was selected as the optimal medium for axillary bud induction in *E. bungeanus*.

Effect of different plant growth regulators kinds, concentrations, and combinations on stem segment induction

To screen for optimal plant growth regulator types, concentrations, and combinations, sterile stem segments were individually inoculated into WPM containing various combinations of plant growth regulators to observe their effects on axillary bud induction. This study used three concentrations of both 6-BA and NAA, resulting in nine experimental combinations (Table 5). The results indicated that most plant growth regulator-treated groups exhibited a significant increase in induction rate. However, when the concentrations of 6-BA and NAA reached their highest levels, the induction rate was lower than that of the control group, indicating an inhibitory effect. Considering the induction rate, average number of induced lateral buds, and growth vigor of buds and shoots, treatment 5 was chosen. Thus, the optimal combination for stem segment induction of *E. bungeanus* was WPM supplemented with 6-BA at 1.0 mg L^{-1} and NAA at 0.2 mg L^{-1} (Fig. 2).

Stem segment subculture

Effect of different media concentrations on stem segment proliferation

Transfer of induced 2–3 cm shoots into different concentrations of WPM culture medium (i.e., WPM, ½ WPM, and ¼ WPM) was conducted to observe their proliferation. Starting from the 20th day, the axillary buds at the base of the shoots began to form shoot clusters, and over time, the number of shoots in the clusters increased.

The experimental results indicated that the growth of shoot clusters improved with a reduction in the content of essential elements in the culture medium. The leaves gradually turned deep green, and the proliferation coefficient reached its highest value of 4.89 (Table 6, Fig. 3). Considering the proliferation coefficient and the growth of shoot clusters, the optimal axillary bud proliferation culture medium for *E. bungeanus* was determined to be ¼ WPM.

Effect of different kinds, concentrations, and ratios of plant growth regulators on axillary bud proliferation

To screen for the optimal combination of plant growth regulator types, concentrations, and ratios, axillary buds with good growth and similar sizes were inoculated into ¼ WPM medium with different plant growth regulator concentrations. The present study, 6-BA and NAA were set at three concentrations, resulting in nine experimental treatments (Table 7). These results indicate that the combination of low concentrations of 6-BA and NAA favors axillary bud proliferation. However, there was no significant difference in the proliferation coefficients between treatments 1 and 2, the axillary buds in the treatment 1 appeared slender with slightly curled leaves and developed a larger callus at the base. In contrast, the axillary buds in the treatment 2 exhibited robust growth, naturally extended leaves, and healthy performance. Considering the induction coefficient and growth of axillary buds, the treatment 2 was selected as the optimal combination for axillary bud proliferation in *E. bungeanus*, consisting of ¼ WPM medium supplemented with 6-BA 2.0 mg L^{-1} and NAA 0.1 mg L^{-1} (Fig. 4).

Rooting culture

Effect of different culture media on rooting

In the present study, robust plants were selected from aseptic seedlings obtained using the abovementioned subculture. Basal segments with 3–5 leaves and 1–2 cm length were excised and inoculated into solid culture media containing IBA (0.1 mg L^{-1}) in ½ MS, MS, ½ WPM, and WPM. The induction of rooting and subsequent growth was meticulously observed. The experimental results revealed a trend in the average root number from the highest to lowest as follows: ½ MS > MS > ½ WPM > WPM (Table 8). This suggests a notable impact of the type and dosage of culture medium on rooting. Selecting an appropriate medium can significantly enhance the propagation efficiency. Notably, the highest rooting rate of 60.00% was achieved on ½ MS medium, with an average of 4.29 roots per plant. The plants exhibited vigorous growth, and their roots were densely packed. Excessive concentrations of macronutrients in the MS medium are unfavorable for root development. However, the lower inorganic salt concentration in the WPM might lead to poor nutrition in the later stages, thereby affecting rooting. Considering both the rooting rate and average root number, the optimal rooting medium was ½ MS medium.

Effects of different rooting agents and concentrations on root formation

In the present study, robust plants were selected from aseptic seedlings obtained using the abovementioned subculture process. Basal segments of 3–5 leaves, 1–2 cm long, were excised and inoculated into solid culture media containing different rooting agents and concentrations of ½ MS. The induction of rooting and subsequent growth was meticulously observed. Two types of roots were observed during the statistical analysis: coarse roots, which were few (1–5), and fibrous roots, which were more abundant. The experimental results revealed that the combination treatments did not perform as well as the single treatments (Table 9). Treatment 2, a combination of two rooting agents, had a significantly higher rooting rate than the other eight groups, reaching 61.67%, with mostly dense fibrous roots. Treatment 10, with a single rooting agent, achieved a rooting rate of 78.33%, showing significant differences from other groups, and formed coarse roots. However, the rooting rate significantly decreased above this concentration. Considering both the rooting rate and root formation conditions, treatment 10 was selected as the optimal combination for root formation and consisted of ½ MS medium supplemented with IBA at 0.2 mg L^{-1} (Fig. 5).

Discussion

Plant tissue culture, based on the theory of cellular totipotency, has been developed as an effective programme for regenerating intact plants, allowing for factory-scale production of plants (Shahzad et al. 2017, Lee and Pijut 2017). The success of plant tissue culture depends not only on the regenerative capacity of different plants but also on the choice of explants, timing of sampling, choice of medium, and combination of growth regulators (Ċosiċ et al. 2015, Raomai et al. 2015, Rout and Sahoo 2007).

Selection of explants

The selection of explants for plant tissue culture primarily follows the principles of ease of manipulation, ease of culture initiation, and rapid seedling development (Kirillov et al. 2022). Opting for shoot-bearing stem segments as explants adheres to the criteria above and presents wide availability, low variability, and uniformity attributes. Thus, in this study, shoot-bearing stem segments were used as the experimental materials.

Sampling time

In this study, we discovered that the sterilization effectiveness of the experimental materials was closely correlated with the timing and season of collection. The collection and sterilization processes were relatively uncomplicated from March to May, characterized by dry air and ample sunlight. However, from June to August, increased rainfall lead to higher humidity, which provided favorable conditions for microbial growth. During this period, stem segments tend to harbor a greater microbial load, making sterilization more challenging. Starting in September, the differentiation capacity of the stem segments began to diminish (Teixeira da Silva and Dobránszki 2013).

Selection of disinfection methods for stem segments

The present study used a combination of two disinfectants to sterilize explants. The initial step involved soaking the explants in 75% ethanol for disinfection, followed by treatment with either NaClO or $HgCl_2$. Ethanol is one of the most commonly used disinfectants for explant sterilization. The optimal bactericidal effect was achieved with 70–75% ethanol. Excessive ethanol concentrations can lead to rapid dehydration and coagulation of proteins on the microbial surface, forming a dry film that impedes ethanol penetration and hampers the intended sterilization effect (Ahmad et al. 2016, Mahmoud and Al-Ani 2016; Gochhayat et al. 2017; Nisa et al. 2019). Another disinfectant was 0.1% $HgCl_2$ with an optimal disinfection time of 7 min. This finding aligns with the stem disinfection method described by Mahmoud and Al-Ani (2016), who observed that prolonged exposure to $HgCl_2$ led to browning and death of stem segments (Mahmoud and Al-Ani 2016; Gochhayat et al. 2017). Similarly, Zhao et al. (2016) observed an increase in explant mortality after extended $HgCl_2$ exposure during *E. alatus* stem disinfection. This is because the substance is prone to residues in plant material. Hence, after the disinfection process, it was essential to rinse with sterile water five times to ensure thorough removal. This liquid presents significant hazards to human health and the environment, necessitating specialized disposal. In conclusion, selecting an optimal sampling time and disinfection method is pivotal for successfully establishing aseptic systems (Mahmoud and Al-Ani 2016). Consistent with the findings of Mahmoud and Al-Ani (2016), the efficacy of NaClO as a disinfectant was lower compared to $HgCl_2$ in this study.

Selection of basal medium

The media used in plant tissue culture are nutrient sources essential for the survival of the explants and contains all the nutrients required for the normal growth and development of plants (Hussain et al. 2012). However, the compositions of different media vary, and the growth responses of different plants and different stages of plant tissues are also different (Bell et al. 2009, Purwito et al. 2021). In the present study, WPM, MS, and B5 media were used. Among them, WPM is used mainly for the regeneration of woody plants, whereas MS is a general multi-purpose medium composed of balanced nutrients. WPM has lower inorganic salt concentrations, and ammonium and nitrate concentrations in MS are higher than in WPM and B5 media (Bell et al. 2009, Kaur et al. 2022) In the present study, among the three basal media mentioned above, WPM with lower inorganic salt concentrations yielded the highest rate of axillary bud induction from *E. bungeanus* stem segments. The induced shoots showed minimal callus formation at the base, deep green leaves, and robust growth. This finding aligns with the optimal basal medium choices reported for *E. alatus* cv. 'Compacta' by researchers such as Bu et al. (2020). During the rooting phase, medium containing medium-strength ½ MS was identified as the optimal choice. The results are consistent with the findings of Ning et al. (2021).

Plant growth regulators

Different types of plant growth regulators, with varying concentrations and ratios, play important roles in the regulation of cell division and differentiation during plant tissue culture. Despite their minute quantities in the culture medium, plant growth regulators play a pivotal role in effectively regulating the growth and development of *in vitro* cultured materials. Extensive studies on plant tissue culture have demonstrated that selecting the appropriate types, concentrations, and ratios of plant growth regulators, and culture conditions is crucial for successful tissue culture experiments.

Cytokinins have the most notable effects in tissue cultures, where they are often used in combination with auxins, to stimulate cell division and control morphogenesis. Among the two growth regulators, auxins inhibit cytokinin accumulation, whereas cytokinins inhibit at least some of the effects of growth hormones (Van Staden et al. 2008).

The optimal combination obtained in the present study for stem segment induction of *E. bungeanus* was WPM + 1.0 mg L^{-1} 6-BA + 0.2 mg L^{-1} NAA, and the optimal combination for axillary bud proliferation was ¼ WPM + 2.0 mg L^{-1} 6-BA + 0.1 mg L^{-1} NAA. Considering the above results, the axillary bud induction medium component has a low concentration of cytokinin (6-BA) and a high concentration of auxin (NAA) as compared to the proliferation medium component. The induction rate of axillary bud reached 87.22%, and the stem segments with apical buds showed vigorous new bud growth and full buds, whereas stem segments with slightly lignified branches in the middle showed relatively weaker growth, slender branches, and yellowing leaves. A combination of high concentrations of cytokinins and low concentrations of auxins is beneficial for axillary bud proliferation, and the proliferation coefficient reached 4.98. The results were similar to the optimal plant growth regulator combination (cytokinin/auxin) for callus induction and adventitious bud proliferation that is adopted in *E. bungeanus* leaf regeneration and propagation systems (Ning et al. 2021). In conclusion, the choice of plant growth regulators in a nutrient medium and their ratio (cytokinin/auxin) are largely dependent on the morphogenetic potentials of the explants employed for in vitro culture (Wang et al. 2023). In tissue culture, the combination of IBA and NAA is reportedly the most extensively adopted hormone combination for rooting (Ullah et al. 2013). However, according to the results of the present study, the combination of IBA and NAA did not yield the optimal rooting rate; conversely, the optimal results were obtained with low concentrations of IBA alone, with a rooting rate of up to 78.33%. Similar results were obtained in Garnem rootstock and *Mniochloa abersend* studies (Zang et al. 2017).

Conclusion

1. The optimal collection time for *E. bungeanus* was mid-April, with a contamination rate of 1.67% and a survival rate of 83.33%. The preferred sterilization method for stem segments involved pretreatment with 75% ethanol for 20 s, followed by 0.1% $HgCl_2$ sterilization for 7 min, resulting in the highest survival rate of 55.00%.
2. The optimal combination of culture media for inducing axillary buds in *E. bungeanus* was WPM supplemented with 1.0 mg L^{-1} 6-BA and 0.2 mg L^{-1} NAA, achieving an induction rate of 87.22%. The most effective medium for axillary bud proliferation was ¼ WPM supplemented with 2.0 mg L^{-1} 6-BA and 0.1 mg L^{-1} NAA, yielding a proliferation coefficient 4.98.
3. The best rooting medium for *E. bungeanus* consisted of ½ MS supplemented with 0.2 mg L^{-1} IBA, resulting in a rooting rate of 78.33%.

Declarations

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None.

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Statements & Declarations

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Competing interests

Kai-Ge Cui: no conflicts of interest.

Hao-Lin Wang: no conflicts of interest.

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Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Cui K.G., Wang H.L., Quan W.Y., and Ye Z.Z. participated in the experiments and data analyses. Cui K.G. and Wang H.L. participated in the data analyses. Cui K.G., Wang H.L., and Zhao C.R. wrote the manuscript and designed research with Cui K.G., Wang H.L., and Zhao C.R. Overall, Cui K.G. and Wang H.L. equally contributed to this work. All authors have read and approved the final manuscript.

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Tables

Table 1 Effects of different sampling times on explant survival

Sampling time	Contamination rate (%)	Mortality rate (%)	Survival rate (%)
mid-March	4.17 ± 1.44 f	31.67 ± 1.44 a	64.17 ± 1.44 b
mid-April	1.67 ± 1.44 f	15.00 ± 2.50 c	83.33 ± 3.82 a
mid-May	14.17 ± 1.44 e	21.67 ± 1.44 b	64.17 ± 2.89 b
mid-June	26.67 ± 1.44 d	17.50 ± 2.50 c	55.83 ± 1.44 c
mid-July	39.17 ± 2.89 c	15.83 ± 1.44 c	45.00 ± 2.50 d
mid-August	80.00 ± 2.50 b	0.83 ± 1.44 d	19.17 ± 1.44 e
mid-September	97.50 ± 0.00 a	1.67 ± 1.44 d	0.83 ± 1.44 f

Note: Values are the mean and standard deviation; the letters in the table indicate significant differences at 0.05 level (the same below).

Table 2 Effect of ethanol disinfection time on the sterilization results of stem segments

Ethanol disinfection time (s)	Contamination rate (%)	Mortality rate (%)	Survival rate (%)
10	57.50 ± 2.50 a	21.67 ± 1.44 b	20.83 ± 1.44 b
20	41.67 ± 2.89 b	17.50 ± 2.50 c	40.83 ± 1.44 a
30	40.00 ± 2.50 b	36.67 ± 1.44 a	23.33 ± 3.82 b

Table 3 Effect of different disinfectants and treatment times on the sterilization results of stem segments

Disinfection	Treatment time (min)	Contamination rate (%)	Mortality rate (%)	Survival rate (%)
1% NaClO	10	100 a	-	-
1% NaClO	20	100 a	-	-
2% NaClO	10	98.33 ± 2.89 a	-	1.67 ± 2.89 e
2% NaClO	20	87.50 ± 2.50 b	5.00 ± 2.50 c	7.50 ± 2.50 d
0.1% $HgCl_2$	5	60.00 ± 2.50 c	7.50 ± 2.50 c	32.50 ± 2.50 c
0.1% $HgCl_2$	6	50.83 ± 1.44 d	10.00 ± 2.50 b	39.17 ± 3.82 b
0.1% $HgCl_2$	7	32.50 ± 2.50 e	12.50 ± 2.50 b	55.00 ± 2.50 a
0.1% $HgCl_2$	8	22.50 ± 2.50 f	25.00 ± 2.50 a	52.50 ± 4.33 a

Table 4 Effects of the different medium on the stem with bud induction

Medium	Induction rate (%)	No. of adventitious buds	Growth status
B5	55.00 ± 5.00 b	1.70 ± 0.15 b	The situation of bud withering is severe, with slow growth and yellow leaves.
MS	55.83 ± 1.44 b	1.02 ± 0.32 c	The bud’s growth is poor, with slow development and light green leaves.
WPM	78.33 ± 3.82 a	2.39 ± 0.64 a	The buds are tender green, growing rapidly, and the leaves are deep green.

B5: Gamborg’s B5; MS: Murashige and Skoog; WPM: woody plant medium

Table 5 Effects of different kinds of plant growth regulators, concentration, and ratio on the stem with a bud induction

Treat.	6-BA (mg L^{-1})	NAA (mg L^{-1})	Induction rate (%)	No. of buds
1	0	0	17.22 ± 3.47 g	0.98 ± 0.25 b
2	1.0	0.1	72.78 ± 1.92 b	1.10 ± 0.02 b
3	1.5	0.1	50.56 ± 3.47 d	1.16 ± 0.18 b
4	2.0	0.1	35.00 ± 1.67 f	1.31 ± 0.25 a
5	1.0	0.2	87.22 ± 2.55 a	1.26 ± 0.47 b
6	1.5	0.2	31.67 ± 4.41 f	0.85 ± 0.02 c
7	2.0	0.2	41.67 ± 2.89 e	1.15 ± 0.13 b
8	1.0	0.3	14.44 ± 2.55 g	1.13 ± 0.26 b
9	1.5	0.3	67.22 ± 2.55 c	0.96 ± 0.11 b
10	2.0	0.3	11.67 ± 4.41 g	0.89 ± 0.05 b

Note: 6-BA: 6-benzylaminopurine; NAA: α-naphthalene acetic acid

Table 6 Effects of medium concentration on the stem with bud propagation

Medium	Proliferation coefficient	Growth status
WPM	3.11 ± 0.14 c	Yellow-green callus tissue is at the base, and the leaves have turned yellow.
WPM	3.52 ± 0.13 b	Yellow-white callus tissue is at the base, along with a few yellow leaves.
WPM	4.89 ± 0.27 a	Yellow-green callus tissue is at the base, and the leaves are deep green.

WPM: woody plant medium

Table 7 Effects of different plant growth regulators, concentration, and ratio on axillary bud proliferation

Treatment	6-BA (mg L^{-1})	NAA (mg L^{-1})	Proliferation coefficients	Growth status
1	1.0	0.1	5.03 ± 1.02 a	There is a small amount of yellow-green callus tissue at the base, the seedlings are weak, the leaves are deep green, and the leaves are small.
2	2.0	0.1	4.98 ± 1.13 a	There is a small amount of yellow-green callus tissue at the base. The seedlings are robust, with deep green leaves and large upper leaves.
3	3.0	0.1	4.33 ± 1.02 a	There is a small amount of yellow-green callus tissue at the base. The seedlings are robust, with deep green leaves and large upper leaves.
4	1.0	0.2	3.53 ± 1.00 b	There is a small amount of light yellow-green callus tissue at the base. The seedlings are weak, exhibiting some yellow leaves. The leaves are relatively small.
5	2.0	0.2	1.32 ± 0.04 c	The callus tissue at the base is very small. The seedlings are small, and the leaves are yellow with a slight curl.
6	3.0	0.2	3.32 ± 1.28 b	There is a small amount of yellow-green callus tissue at the base. The seedlings are robust, with deep green leaves and relatively large upper-middle leaves.
7	1.0	0.3	3.11 ± 2.46 b	There is pea-sized dark green callus tissue at the base. The seedlings are weak, with yellow-green leaves that are narrow and small.
8	2.0	0.3	4.91 ± 1.05 a	There is a small amount of yellow-green callus tissue at the base. The seedlings are robust, with some yellow leaves shedding.
9	3.0	0.3	4.34 ± 2.21 a	There is a small amount of greenish-black callus tissue at the base. The seedlings are weak, with some yellow leaves shedding and narrow and small leaves.

6-BA: 6-benzylaminopurine; NAA: α-naphthalene acetic acid

Table 8 Effect of *Euonymus bungeanus* on different kinds of rooting medium

Medium	No. of root	Rooting rate (%)	Growth status
MS	4.29 ± 0.21 a	60.00 ± 4.33 a	The roots are thick and robust, and the plants are healthy and vigorous.
MS	3.26 ± 0.04 b	45.00 ± 5.00 b	The roots are thin and weak, appearing whitish and loosely attached.
WPM	2.56 ± 0.04 c	35.83 ± 5.20 c	The roots are thin and weak, exhibiting a whitish appearance.
WPM	1.38 ± 0.08 d	23.33 ± 3.82 d	The roots are thin and weak, and the base of the plant exhibits a blackish appearance.

MS: Murashige and Skoog; WPM: woody plant medium

Table 9 Effect of different kinds of rooting agent and concentration on rooting

NAA: α-naphthalene acetic acid; IBA: indole-3-butyric acid

Figures

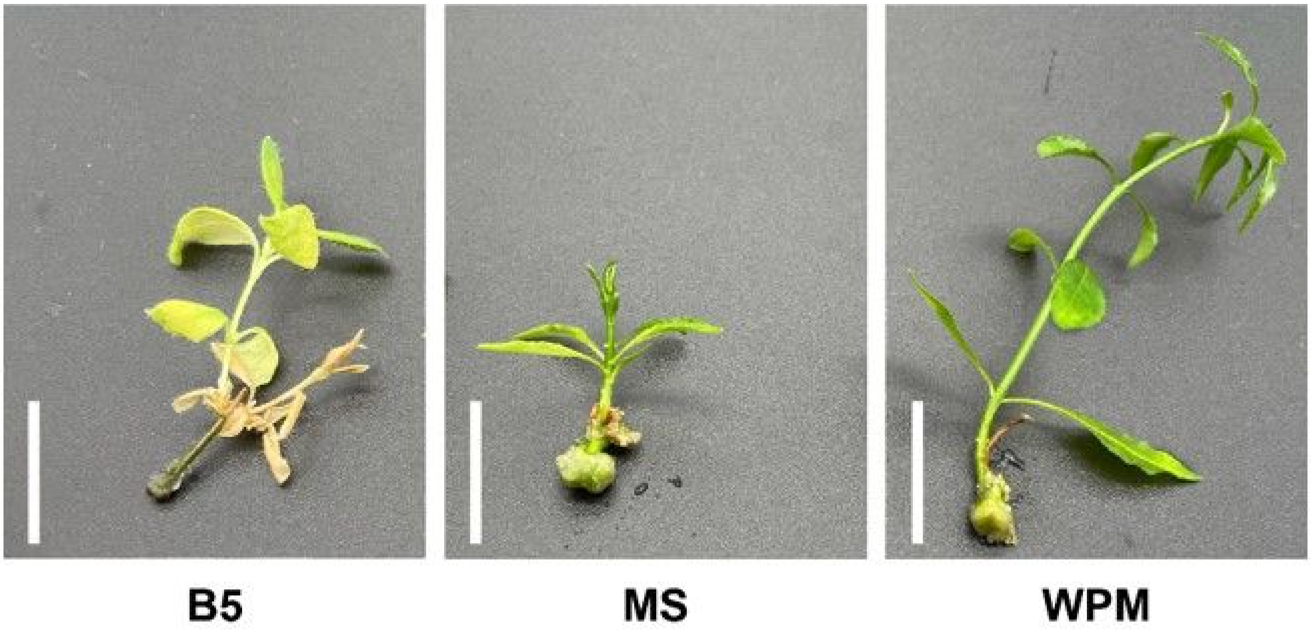

Figure 1

Growth situation of the stem with a bud in the different induction medium after 30 d

B5: Gamborg's B5; MS: Murashige and Skoog; WPM: woody plant medium; scale bar indicates 1.0 cm

Treatment	NAA (mg L^{-1})	IBA (mg L^{-1})	Rooting rate (%)	Growth status
1	0.05	0.2	50.83 ± 2.89 c	There are few fibrous roots. The callus tissue is yellowish-white. The plants are relatively healthy, and the leaves have a bluish-green color.
2	0.05	0.4	61.67 ± 3.82 b	All roots are fibrous in small quantities. The callus tissue is yellowish-white. The leaves are green, and the plants are relatively healthy.
3	0.05	0.6	36.67 ± 3.82 d	No fibrous roots; roots are slightly thick. The callus tissue is yellowish-white and relatively small in volume. The plants are healthy, and the leaves have a normal color.
4	0.1	0.2	51.67 ± 6.29 c	All are fine and short fibrous roots. Callus tissue is predominantly yellowish-white, with a relatively small size. Leaf color appears normal, but the plants show slight signs of weakness in growth.
5	0.1	0.4	57.50 ± 5.00 c	All are fine and short fibrous roots. The base exhibits yellowish-white callus tissue, and the leaves are also yellowish-white.
6	0.1	0.6	23.33 ± 3.82 f	Both primary and lateral roots are present. Yellowish-white callus tissue with a small volume can be observed at the base. The leaves exhibit a light yellow-green color. The plant appears somewhat weak.
7	0.2	0.2	24.17 ± 7.22 f	All roots are adventitious and rootlets. The volume is not substantial, and yellowish-white callus tissue is at the base. The plants appear relatively weak. Some leaf loss is visible but not extensive, primarily showing a light yellow-green color.
8	0.2	0.4	10.00 ± 2.50 g	There are some primary roots without adventitious roots (in small quantities). Yellowish-white callus tissue is present at the base, with a small volume. The plants are not very vigorous, predominantly yellow-green leaf color, and have significant leaf loss (more than 50%).
9	0.2	0.6	-	No roots are present. Occasionally, small, semi-transparent yellowish-white callus tissue is observed. The plants in this group have significantly poorer growth than the other groups, with most leaves falling off.
10	-	0.2	78.33 ± 8.04 a	The roots are relatively thick and robust, with longer adventitious roots present. The plants appear to be healthier and more vigorous. The leaves exhibit a darker green color. The callus tissue at the base of the stem is light yellow-brown.

11	-	0.4	40.00 ± 5.00 d	The roots are thick and strong, and no visible rootless plants exist. At the base of the stem, there is yellow-white, semi-transparent callus tissue, and a small number of leaves appear to have fallen off.
12	-	0.6	31.67 ± 3.82 e	The roots vary in thickness, with some being thicker and others thinner. They are relatively short and radiate outward. The overall color is mainly green. A few leaves can be observed to have fallen off.
13	0.2	-	10.83 ± 3.82 g	The roots are thick. There is a small amount of yellow-white, semi-transparent callus tissue. The upper part of the plant shows better growth, while the lower part exhibits some yellowing and leaf loss.
14	0.4	-	5.83 ± 3.82 h	The roots are thick. There is only a small amount of loose, yellow-white, semi-transparent callus tissue. The upper part of the plant shows better growth, while the lower part exhibits some yellowing and leaf loss.
15	0.6	-	-	No roots are present. Large-sized callus tissue has formed, mostly semi-transparent and loose in structure. The plant shows severe leaf loss. The growth is stunted and not extended, with the upper leaves being predominantly dark green.

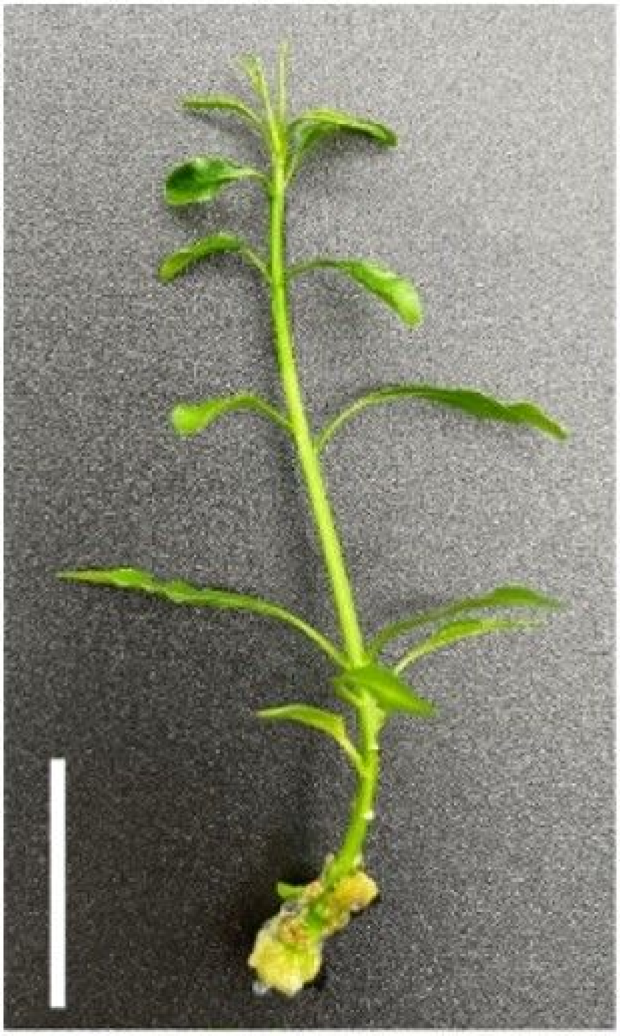

Figure 2

Growth situation of the stem with a bud in the best induction medium after 30 d; scale bar shows 1.0 cm

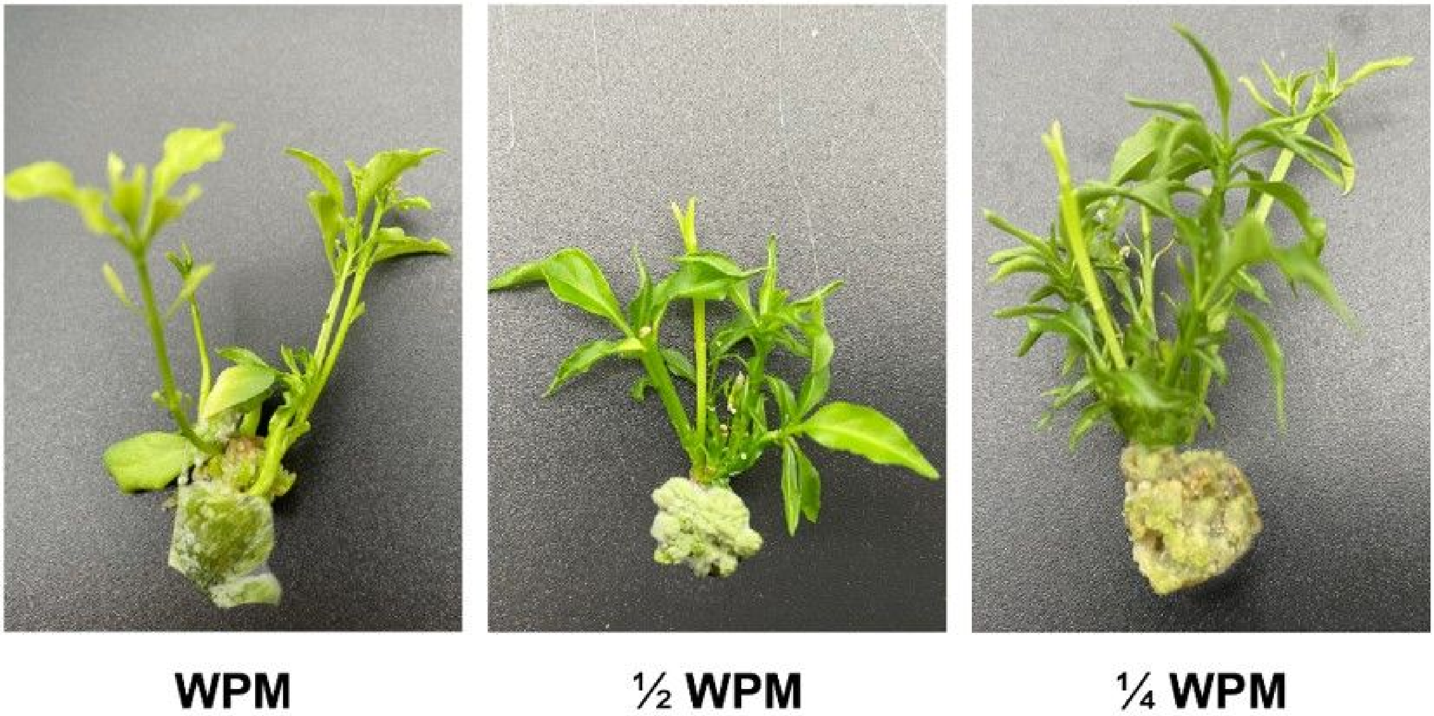

Figure 3

Growth situation of the stem with a bud in different concentration propagation medium

WPM: woody plant medium

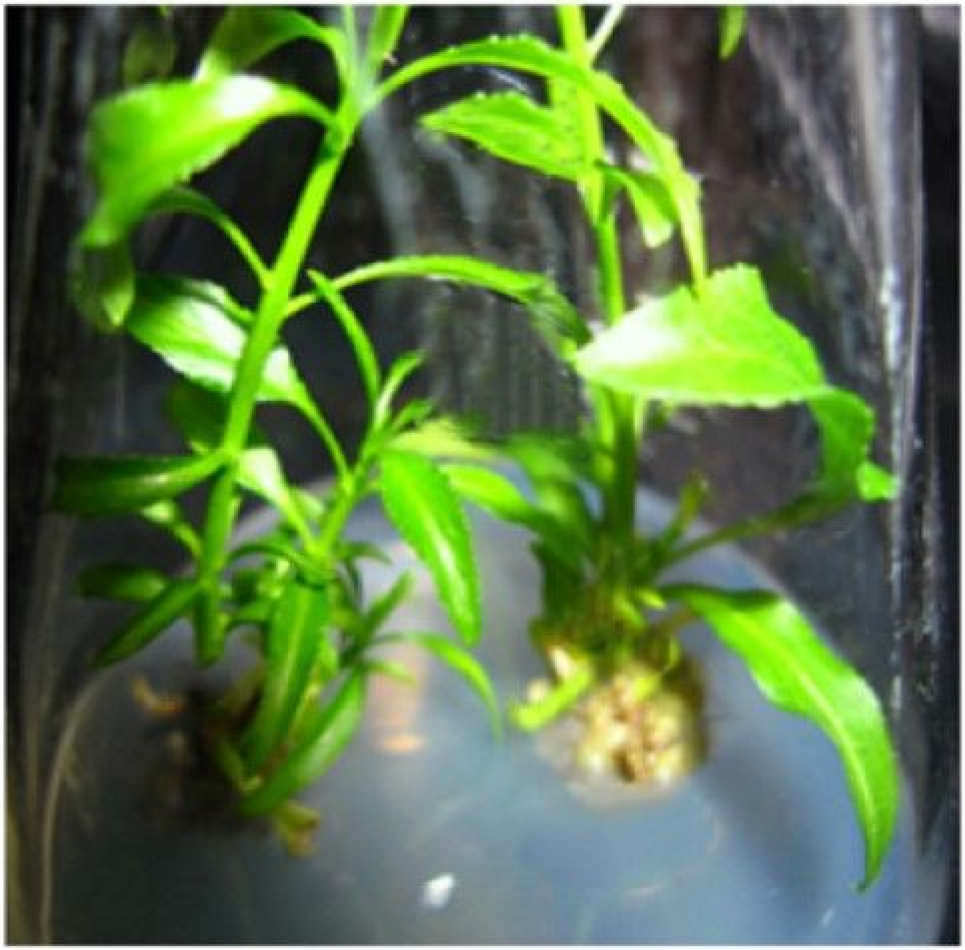

Figure 4

Growth situation of the stem with a bud in the best proliferation medium after 40 d

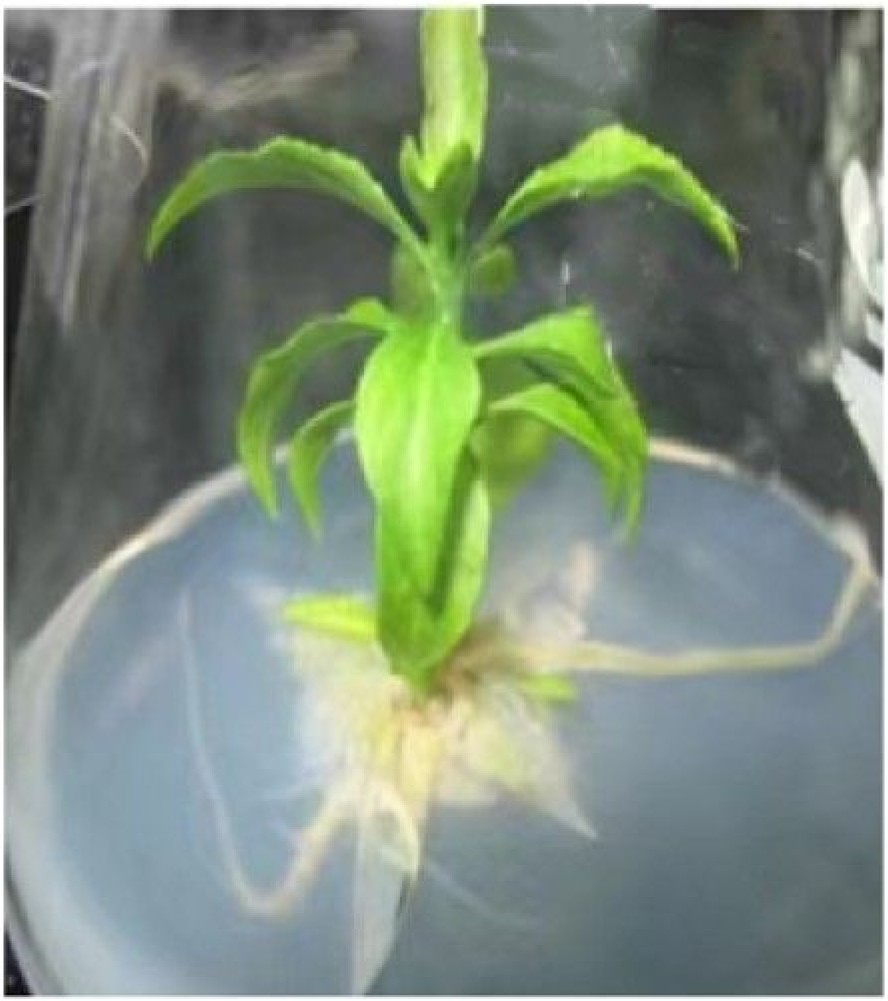

Figure 5

Growth situation of rooting culture in the best rooting medium after 40 d